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Suppression of mammalian bone growth by membrane transport inhibitors.

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ABSTRACT (250 words max)

Bone lengthening during skeletal growth is driven primarily by the controlled enlargement of growth plate chondrocytes. The cellular mechanisms are unclear but membrane transporters are probably involved. We investigated the role of the Na^+/H^+ antiporter (NHE1) and anion exchanger (AE2) in bone lengthening and growth plate hypertrophy in P7 rat bone rudiments using the inhibitors EIPA (5-(N-ethyl-N-isopropyl)amiloride) and DIDS (4,4-diidothiocyano-2,2-stilbenedisulphonate) respectively. We have also determined cell-associated levels of these transporters along the growth plate using fluorescent immunohistochemistry (FIHC). Culture of bones with EIPA or DIDS inhibited rudiment growth (50% at approx. 250μM and 25μM respectively). Both decreased the size of the hypertrophic zone ($P<0.05$) but had no effect on overall length or cell density of the growth plate. *In situ* chondrocyte volume in proliferative and hypertrophic zones was decreased ($P<0.01$) with EIPA but not DIDS. FIHC labelling of NHE1 was relatively high and constant along the growth plate but declined steeply in the late hypertrophic zone. In contrast, AE2 labelling was relatively low in proliferative zone cells but increased ($P<0.05$) reaching a maximum in the early hypertrophic zone, before falling rapidly in the late hypertrophic zone suggesting AE2 might regulate the transition phase of chondrocytes between proliferative and hypertrophic zones. The inhibition of bone growth by EIPA may be due to a reduction to chondrocyte volume set-point. However the effect of DIDS was unclear but could result from inhibition of AE2 and blocking of the transition phase. These results demonstrate that NHE1 and AE2 are important regulators of bone growth.

INTRODUCTION

The growth plate comprises a relatively thin layer of cells (chondrocytes) arranged in columns which lie perpendicular to, and are entirely responsible for, new bone formation during longitudinal growth of the skeleton. The chondrocytes are highly organised with a 'reserve zone' preceding the proliferative zone chondrocytes (PZC) which are relatively small and flattened. However after a specific time period, differentiation of the chondrocytes occurs leading to a dramatic increase in volume and the formation of hypertrophic zone chondrocytes (HZC). The cell volume which can increase dramatically from 1000μm^3 to 10,000μm^3 (Bush *et al*., 2008b), is mainly in the longitudinal axis, and accounts for 60-80% of bone lengthening (Wilsman *et al*., 1996a,b). There is a positive linear relationship between the rate of longitudinal bone growth over 24hrs and the final volume of hypertrophic chondrocytes (Breur *et al*., 1991).

The increase in cell volume occurs by both cell swelling (increasing water content of cells; Bush *et al*., 2008b) and also classical hypertrophy (increase in cell mass; Buckwalter *et al*., 1996) however the mechanism which drives the enlargement of growth plate chondrocytes is poorly understood. The controlled increase in chondrocyte size and the regulation of the intracellular environment (e.g. ion content, pH_i) to permit optimal metabolism must be closely regulated throughout the hypertrophic process mainly by the activity of the membrane transporters in the chondrocyte membrane. It is likely that there is an important role for transporters which regulate the movement of Na^+ and anions (e.g. HCO_3^-) across the cell membrane as these are known to be essential for the control of cell volume and pH_i in a wide range of cell types (Hoffmann *et al*., 2009).

Previous studies have shown a role for the NKCC cotransporter (NKCC1) in mediating growth plate chondrocyte hypertrophy (Bush *et al*., 2008b). Incubating growing metatarsals/metacarpals from P7 rats in the presence/absence of the specific inhibitor bumetanide demonstrated a 35% inhibition of bone growth suggesting that other – as yet unidentified - membrane transporters could also promote chondrocyte hypertrophy and thus bone growth. The two other transporters that could potentially be involved because of their contribution to cell volume and pH_i regulation, are the Na^+/H^+ exchanger (NHE) and the anion exchanger (AE) and it is the purpose of this study to clarify their possible role in bone growth.

Since the discovery of the Na^+/H^+ exchanger, 9 NHE isoforms have been identified. NHE1 was the first identified and is distinct in that it is ubiquitously expressed in all mammalian cells and plays a 'housekeeping' role in the regulation of pH_i and cell volume (Malo & Fliegel, 2006). This isoform was the

most sensitive to amiloride and its derivatives (e.g. EIPA; 5-(N-ethyl-N-isopropyl)amiloride; Masereel *et al*., 2003). NHE1 is the key regulator of pH_i in the majority of articular chondrocytes within hyaline cartilage (Tattersall & Wilkins, 2008) however cells within the superficial zone utilise a HCO_3^- - dependent process (Simpkin *et al*., 2007). Increased activity of NHE1 combined with the parallel activation of the anion exchanger isoform AE2 occurs following the shrinking of cells in media of raised osmolarity. This gives rise to the recovery of cell volume through the process of regulatory volume increase (RVI) and also the maintenance of pH_i in the face of the osmotic challenge (Shen *et al*., 2002; Liu *et al*., 2011). Activation of these transporters *without* a change in extracellular osmotic pressure, will directly increase cell volume and re-set pH_i (Hoffmann *et al*., 2009) and therefore could be a mechanism utilised by growth plate chondrocytes to drive cell enlargement and maintain pH_i. during hypertrophy.

This study therefore extends previous work by testing the hypothesis that NHE1 and AE2 are involved in the regulation of longitudinal growth in mammalian long bones. We incubated metatarsal rudiments from P7 rats in the presence of EIPA or DIDS ((4,4-diidothiocyano-2,2-stilbenedisulphonate; a relatively non-specific inhibitor of AE2), and observed almost complete inhibition of bone growth. The height of the hypertrophic zone (HZ) of the growth plate in the presence of these drugs was significantly reduced, but there was no change in cell density or viability. We found that with DIDS there was no significant change to the volume of *in situ* PZ or HZ chondrocytes, however with EIPA treatment, there was a significant reduction cell size. Using fluorescent immunohistochemistry (FIHC), we observed relatively high levels of cell-associated NHE1 labelling which declined in the latter stages of hypertrophy. In contrast, AE2 labelling was initially low, but increased significantly until early hypertrophy, and then decreased significantly at the final stages of chondrocyte hypertrophy. These results suggest that both transporters are essential for bone growth.

MATERIALS AND METHODS

Biochemicals and solutions. Biochemicals were obtained from Invitrogen Ltd., (Paisley, U.K.) unless otherwise indicated. The following culture media were used: (a) Dissection media which comprised PBS with 7.5% (v/v) α-modified essential medium (α-MEM) and 2% (w/v) bovine serum albumin V (300mOsm; pH 7.4). (b) Standard culture media which contained α-MEM supplemented with: Na_2 glycerol bi-phosphate (1mM); bovine serum albumin (BSA, Fraction V, 0.2% (w/v) 5mg.ml^{-1}; L-ascorbic acid 1% (v/v); penicillin and streptomycin (final concentrations of 100IUml^{-1} and 100µg.ml^{-1} respectively) (300mOsm; pH 7.4). The anion exchange (AE) inhibitor DIDS (4,4-diidothiocyano-2,2-stilbenedisulphonate) was prepared fresh for each experiment as a 100mM stock solution in 0.1M $KHCO_3$. The Na^+/H^+ exchange inhibitor EIPA (5-(N-ethyl-N-isopropyl)amiloride) was prepared as a 120mM stock solution in DMSO. The antibodies used were rabbit polyclonals to NHE1 (ab67313, Abcam, Cambridge, U.K.) and AE2 (ab42687, Abcam, Cambridge, U.K.).

Animals, bones and growth plate preparation. Sprague-Dawley 7-day-old rat pups (P7) were humanely killed for other experiments following Home Office guidelines. Rudiments from the middle three metatarsals with intact growth plates were carefully removed and temporarily placed in dissection medium. For the *ex vivo* cultures, the overall length of the rudiments were measured using an eyepiece camera (DCM35 Pixel Digital Eyepiece Camera, Brunel Microscopes, U.K.) fitted to a stereomicroscope (Wild M3, Switzerland). An image of a rule was acquired to provide calibration, and bone lengths measured (to 0.1mm) using ImageJ software (NIH, Bethesda, Maryland, U.S.A.). All results were expressed as a percentage change from the length at day 0. Rudiments were then incubated in standard culture media (5% CO_2, 95% air, pH 7.4, 37^{o}C) supplemented with the drugs as described in Result. These bones were chosen since at P7, bone mineralisation was at a minimum and the preparation of histological sections could be performed by avoiding the possible immuno-compromising bone decalcification step.

Preparation of growth plates for histology. Bones were fixed overnight in 1.3% glutaraldehyde (GA) with 0.5% ruthenium hexamine trichloride (RHT; pH 7.4) with osmolarity adjusted to 300mOsm to avoid fixative-induced artefacts to chondrocyte shape/volume (Loqman *et al*., 2010). Bones were then dehydrated through a series of ethanol solutions and embedded in paraffin wax using a standard procedure (Kiernan, 1999). Bones were then cut into longitudinal 10µm serial sections (Reichert-Jung Microtome 2050 Supercut, Arnsberg, Germany). After de-paraffinisation with xylene and rehydration with a series of ethanol solutions

(100%, 90%, 75%), the sections were stained with 0.1% toluidine blue O in PBS (pH 5.5; 30secs at 21^{o}C) using the technique adapted from Bancroft and Cook, (1994). The sections were then mounted on poly-l-lysine-coated microscope slides (Polysine^{TM}, VWR International, Leicestershire, U.K.) rinsed briefly in distilled water, and air dried prior to mounting with Fluorosave^{TM} and cover slips.

Measurement of growth plate length and cell density. Images of distal growth plates were taken using the transmitted light detector of an upright confocal laser scanning microscope (Zeiss LSM510) fitted with a x10 dry objective lens. To determine the beginning and terminal points of the GP, several subjective criteria based on cell size and organization were used as described (Hunziker, 1994). The GP length and the late HZ height were then identified and measured using an established procedure (see Bush *et al*., 2010). Briefly, three zones were identified by eye and marked by drawing a horizontal freehand line along the top of the proliferating cell border. The clear demarcation of cell enlargement between proliferative and hypertrophic regions and the zone of mineralization at the base of the hypertrophic zone was evident. Another straight line was drawn perpendicularly along the mid GP section (Zeiss LSM Image Browser Software, Carl Zeiss MicroImaging GmbH, Germany) (see Fig. 2). The GP length between the two points where the vertical and horizontal lines met most proximally and distally, was measured using ImageJ software (NIH, Bethesda, Maryland, USA). The late HZ height was determined from the distance between the two points where the lines met at the PZ-HZ cell enlargement regions proximally and the zone of mineralization distally. The total number of cells within the late hypertrophic zone was counted by eye, and the surface area of the zone was selected using freehand selection and measured using ImageJ. The cell density was determined based on the total cell numbers counted over the measured area of the late HZ (cells/mm^2).

Fluorescent labelling of *in situ* growth plate chondrocytes. For the analysis of GPC volume viability, bones were incubated in standard culture medium supplemented with inhibitors at the following concentrations; (i) 0μM DIDS ($KHCO_3$ vehicle alone), (ii) 250μM DIDS, (iii) 0μM EIPA (DMSO vehicle alone), and (iv) 444μM EIPA. Bone rudiments were maintained at 37^{o}C for 24hrs as described above. Bones were then washed in fresh culture media before being bisected sagitally, and incubated with CMFDA, and PI (both at 5μM; 60mins; 37^{o}C). CMFDA is permeable to viable cells, and in the cytoplasm is metabolised by cellular esterases to produce a membrane-impermeant fluorescent product that stains the cytoplasm of viable cells green. PI however is only capable of crossing plasma membranes of dying/dead cells and stains the nuclei of the dead cells red (Amin *et al.*, 2008). Bones were then fixed in 4% (v/v) paraformaldehyde

overnight, and stored at 4°C in PBS until visualised by CLSM. The analysis of chondrocyte volume was performed using 3D imaging software (Volocity®, Improvision, Coventry, UK) on scanned CLSM images using the calibrated cell volume method as previously described (Loqman *et al*., 2010). Chondrocyte viability was evaluated by estimating the proportion of PI-labelled chondrocytes compared to the total labelled cell population (CMFDA + PI-labelled) within comparable regions of interest (ROIs) in the growth plates of bones cultured under the different conditions. For experiments on living *in situ* chondrocytes, GPs were incubated with calcein-AM and PI (30mins; 5μM each; 37°C) and imaged as described (Loqman *et al*., 2010).

Fluorescent immunohistochemistry (FIHC). On each slide, typically 3-4 sections were designated as negative controls while the other 2-3 underwent normal Ab labelling. Non-specific Ab sites were blocked using goat serum (1:5 dilution; 30mins; 21°C) and then replaced by the specific 1° antibodies (Abs) under investigation. Optimal dilution of the 1° Ab was determined using a series of different titres (Renshaw, 2007). For negative control sections, the 1° Ab was omitted and replaced with the serum from the species from which the 2nd Ab was generated. Slides were then incubated overnight at 4°C in a humidity chamber, then washed 3x (5mins each) with PBS before being incubated with a FITC (fluorescein isothiocyanate)-tagged 2° Ab at 37°C for 1hr. Sections were then washed 3x (5mins each) with PBS. Next, coverslips were mounted using Fluorosave™, slides wrapped in aluminium foil and left to dry (3hrs; 4°C) prior to imaging by CLSM. In preliminary experiments, antibodies against the following transporters were tested on growth plate chondrocytes: AE1 (sc-20559; Santa Cruz Biotechnology Inc., Santa Cruz, U.S.A. control tissues tested were rat red blood cells and kidney); AE3 (LSC20639; LifeSpan BioSciences Inc., Seattle, U.S.A.) control tissue tested was rat kidney). No positive immunostaining for either AE1 or AE3 were detected on growth plate chondrocytes whereas strong cell specific labelling was observed for control tissues suggesting that AE2 was the major isoform in these chondrocytes.

Measurement of NHE1 and AE2 cell-associated fluorescent labelling. In order to measure cell-specific immunofluorescence staining intensity of individual chondrocytes, cells along the length of the GP were visualised at high magnification (x63 objective) by CLSM. GP images in sequential zones were then 'stitched' together to produce a montage of the whole length of a GP from the proliferative to hypertrophic zone. Each imaged growth plate was divided into 8 equal sections (S1-S8) as described (Loqman *et al*.,

2010). The average fluorescence labeling intensity associated with AE2 or NHE1 Abs in individual cells was determined along the GP in an unbiased semi-quantitative manner using ImageJ Java-based scientific image processing software as described (Bush *et al*., 2010).

Confocal Laser Scanning Microscopy (CLSM). An upright Zeiss Axioskop LSM 510 (Carl Zeiss Ltd., Welwyn Garden City, Herts., U.K.) was used to acquire fluorescent images of *in situ* GPC. Cells were visualized using a Plan-Neofluar x10 dry objective (N.A.=0.3) for low power overviews and an Achroplan x63 ceramic water-dipping lens (N.A.=0.95) for high magnification views. Fluorescent-labelling of intact cells was performed by visualising intracellular calcein or CMFDA excited using an Argon laser (488nm) and emitted fluorescence detected at 517nm using a 500-550nm band pass filter. The DNA of dead cells was observed using propidium iodide (PI) excited using a He-Ne laser (543nm) and emitted fluorescence detected using a 560nm long pass filter. The pinhole was set at 1.00 Airy unit, and optimal imaging quality determined by varying laser power and detector sensitivity. The scanning speed was typically 0.6Hz with 2 frame integration of a 512x512 pixel image, with serial 1μm z-step optical sections (see Bush & Hall, 2001a,b; Bush *et al*., 2007 for details).

Presentation of results and statistical analysis of data. Data were expressed as means ± S.E.M. from a minimum of 3 bones from (n) separate animals and (N) chondrocytes at each condition with data shown as (n[N]). Statistical significance between two groups was evaluated using two-tailed Student's unpaired *t*-tests, with trends in data sets analysed using a one-way ANOVA. Statistical tests were performed using SigmaPlot® (ver. 11.0; Systat Software Inc., GmbH, Germany) and *P* values considered significant when $P<0.05$.

RESULTS

1. Dose-response inhibition of bone growth by DIDS or EIPA. In order to study the roles of AE and NHE on bone lengthening, dose-response experiments were performed (Fig. 1a,b). The mean increase in bone length from the initial value in the DIDS control medium was 6.2±1.3% (n=4) over 24hrs (Fig. 1a). Exposure to increasing DIDS concentrations up to 1mM resulted in dose-dependent reduction of bone lengthening. This was most dramatic at 50μM DIDS, with bone lengthening significantly reduced to 1.9±0.9% (n=4; $P<0.05$). All DIDS concentrations resulted in a significant reduction in longitudinal growth ($P<0.05$; n=4). A DIDS concentration of 250μM appeared to give maximal inhibition and was therefore used for subsequent studies. Fig. 1b illustrates the effect of EIPA (0-1.3mM) on metatarsal bone longitudinal growth. The mean increase in length after 24hrs for control bones was 7.9±0.5% (n=36) and although the drug vehicle was different ($KHCO_3$ *vs* DMSO for DIDS and EIPA respectively) the difference was not significant ($P>0.05$). EIPA resulted in a dose-dependent decrease in bone lengthening, but only became significantly reduced at an EIPA concentration at 111μM and above ($P<0.05$). Whilst an EIPA concentration of 1.3mM showed maximum reduction of bone lengthening (1.4±0.3%), 444μM was the considered to be the concentration that produced the optimal reduction of bone lengthening and was therefore used in the later experiments. The concentrations of DIDS or EIPA resulting in a 50% inhibition were approximately 25μM and 250μM respectively. For both drugs, there was a significant decrease in overall bone longitudinal growth inhibition as the DIDS or EIPA concentrations were increased (one-way ANOVA;$P<0.001$).

There might be concern that these drugs could induce relatively non-specific cell death leading to an apparent inhibition of bone lengthening. Accordingly a cell viability test was performed on the GPC of metatarsal bones after 24hrs of treatment with 250μM DIDS or 444μM EIPA with appropriate controls. Bones were then bisected and incubated with CMFDA and PI and the cells visualised by CLSM (see Materials and Methods). There was no difference in the proportion of live:dead chondrocytes in any of the samples (data not shown) suggesting that these drugs were not inhibiting bone lengthening by causing the death of GPC.

2. Changes to GP length, late HZ height and cell density following treatment with DIDS or EIPA. Having recorded the dose-response inhibition by DIDS or EIPA on *ex vivo* bone longitudinal growth (Fig.1), the histological changes were examined. Emphasis was placed on the GP HZ cells, as the size of these cells

is a determinant of the rate of longitudinal bone growth (Wilsman *et al*, 1996b). The total length of the GP, the height of the late HZ and the cell density in the late HZ were measured (see Materials and Methods; Fig. 3). Table 1 shows the results of the metatarsals cultured in the presence or absence of DIDS (250μM) or EIPA (444μM). There was no significant difference in the total growth plate length between the control and DIDS-treated group (unpaired Student's t-Test; $P>0.05$; n=4-6). Similarly there was no significant difference between the control and EIPA-treated group ($P>0.05$). The height of the HZ in the DIDS-treated GP was also not significantly different from the untreated bones ($P>0.05$). Likewise, there was no significant difference between the height of the HZ in the control compared to the DIDS-treated GP ($P>0.05$). However, when HZ height was expressed as a percentage of the total GP length, the relative HZ height in the DIDS and EIPA-treated groups were significantly less compared to the controls ($P<0.05$; n=4-6). This suggests that in the presence of DIDS or EIPA, the reduction in the heights of the HZ over the length of the GP could account for the perturbation of normal GPC hypertrophy and thus bone lengthening by these drugs.

The reduction in the size of the late HZ height over the total GP length could be due to a decrease in cell density (*e.g.* caused by cell death), and/or changes in GPC size (*e.g.* caused by perturbation of normal cell enlargement). To further investigate this, the cell density in the HZ was measured. The results (Table 1) showed no difference of GPC in the HZ of the DIDS or EIPA-treated metatarsals compared to the controls ($P>0.05$) suggesting that these drugs did not cause significant loss/death of hypertrophic GPC.

3. Effect of DIDS or EIPA on the volume of *in situ* GPC. A more likely action of these drugs on bone lengthening could be by inhibiting the increase in cell volume occurring with hypertrophy. The effects of DIDS or EIPA on *in situ* GPC volume were therefore determined using fluorescently-labelled *in situ* chondrocytes and CLSM. Volume analysis was performed at the PZ (S1-P2) and the HZ (S7-S8; see Materials and Methods). Using a high power objective lens, no difference was observed in the volume of the GPC treated with DIDS and the control (Fig. 3A,B). However there was a clear reduction in cell size between the EIPA-treated GPC and the control (Fig. 3C,D). Quantitative analysis of cell volume was also performed on the GPC in the PZ and HZ from the metatarsal bones of each treatment group (Table 2). The results showed the mean volume of the control cells in PZ was not significantly different from DIDS-treated cells ($P>0.05$). Likewise, the mean volume of the control cells in the HZ was not significantly different from the mean GPC volume in DIDS-treated cells ($P>0.05$). On the other hand, there was significant decrease in the volume of the EIPA-treated cells in the PZ and the HZ compared to the control cells (unpaired Student's

t-Test; $P<0.05$; n=3-6; Table 2). Taken together, these data suggest that the DIDS treatment resulted in no significant changes to the size of the PZ cells and HZ cells whereas EIPA treatment resulted in a significant reduction in size of the these cells.

4. Fluorescent immunohistochemistry of NHE1 and AE2 in P7 rat GPC. Low power CLSM images showed no detectable NHE1 or AE2-associated fluorescence staining in the negative control sections (data not shown) whereas with the GP sections, there was the suggestion of differential labelling for both transporters along the GP (Fig. 4). For NHE1, the fluorescent signal was distinct in the early PZ and was maintained through the late PZ and early HZ, before clearly decreasing in the middle to late HZ. For AE2, the fluorescence signal was low in the early PZ but clearly increased in the late PZ and remained elevated in the early HZ. To examine the overall semi-quantitative distribution pattern of NHE1 and AE2 in GPCs along P7 proximal tibia GP (S1-S8), the average fluorescence labelling intensity associated with membrane transporter localisation was determined (see Materials and Methods). Fig. 5 shows the relatively high level of NHE1 immunofluorescence throughout S1-S5 before decreasing from S6-S8 (one-way ANOVA; $P<0.001$). The fluorescence intensity was significantly lower in S8 compared to the maximum intensity that was reached in S5 (Student's unpaired *t*-test; $P<0.001$). For AE2, there was a significant increase in immunofluorescence intensity of AE2 throughout S1-S5 (one-way ANOVA; $P<0.001$). The peak intensity was observed in S5, which was significantly higher than the original intensity recorded in S1 (Student's unpaired *t*-test; $P<0.001$). After reaching the highest fluorescence intensity in S5, there was a significant decrease in the intensity from S6-S8 (one-way ANOVA; $P<0.001$). The fluorescence intensity of AE2 in S8 was significantly lower than in S1 (Mann-Whitney test; $P=0.008$). Although immunofluorescence labelling of both NHE1 and AE2 shared the highest intensity in S5 and the lowest in S8, the intensity of NHE1 in S1 was significantly higher than in AE2 (Student's unpaired *t*-test; $P<0.005$). Fig. 5 (inset) demonstrates a direct comparison between NHE1 and AE2 immunofluorescent intensity along GP with the data from the main Figure being normalized to 100% at S5 (peak intensity in both graphs) to allow comparison. This indicates that while NHE1-immunofluorescent levels appeared higher throughout S1-S5, the AE2-immunofluorescence levels labelling was initially lower, but gradually increasing toward S5. Both NHE1 and AE2-immunofluorescence labelling decreased dramatically after S5 toward S8.

DISCUSSION

The rate of growth of P7 rat bone rudiments was markedly suppressed by EIPA and DIDS, both widely used inhibitors of the membrane transporters NHE1 and AE2 respectively in a range of cell types. The mechanism of inhibition however would appear to be different because although both drugs reduced the size of the HZ in relation to the overall length of the growth plate, EIPA significantly reduced the volume of chondrocytes throughout the length of the growth plate whereas DIDS had no significant effect (Table 2). Fluorescence immunohistochemistry demonstrated that levels of NHE1 remained high in chondrocytes throughout most of the growth plate, but declined in late hypertrophic cells whereas AE2 levels demonstrated a biphasic effect (Fig. 5). Before proposing a role for these transporters in chondrocyte hypertrophy, it is important to consider potentially confounding effects which might complicate interpretation of the results.

The first issue is the specificity of the drugs used in this study. EIPA is a highly potent analogue of amiloride (Masereel *et al*., 2003) and a very effective inhibitor of NHEs in chondrocytes (Wilkins *et al*., 2000; Simpkin *et al*., 2007), with no reported inhibitory effects on other chondrocyte membrane transporters. However, DIDS is a relatively non-specific inhibitor of AEs (Cabantchik & Greger, 1992; Kidd & Thorn, 2000), as it also blocks Cl^- channels in a range of cell types (Okada, 1997) although at higher concentrations than those used here. DIDS does not however inhibit Cl^- channels in rabbit articular chondrocytes (Sugimoto *et al*., 1996). DIDS at 100μM inhibits AE activity by >90% and bone growth by approx. 85% (Fig. 1A) but has only a minor effect (<10%) on volume-sensitive anion channels in cell types other than chondrocytes (Hallows *et al*., 1994) and therefore an inhibitory effect on growth plate chondrocyte Cl^- channels is unlikely to explain the inhibition of bone growth. DIDS has also been reported to block diastrophic dysplasia sulphate transport (DTDST)-mediated SO_4^- uptake which is essential for proteoglycan synthesis (Satoh *et al*., 1998). However the concentration required (1mM) is substantially higher than that required for inhibition of bone growth in the present study (Fig. 1A). Therefore EIPA would appear to give an accurate assessment of the role of NHEs in bone growth, and although DIDS is less specific, it is probable that the major effect of this drug on bone growth is mediated by inhibition of AE2s.

It was necessary to raise the concentrations of drugs to relatively high levels in order to demonstrate the inhibitory effect on bone growth probably because of their relatively poor permeation through the connective tissue surrounding the bones to the growth plate chondrocytes. The concentrations of drugs which

finally reach the chondrocytes within the growth plates will be markedly less than the levels used in the culture medium (Fig. 1A,B). There was however concern that the drugs might simply be killing chondrocytes through a non-specific lethal action causing an apparent inhibition of bone growth. Nevertheless, we found that even after 24hrs incubation with DIDS (250μM) or EIPA (444μM), there was no noticeable decrease in chondrocyte viability or density suggesting that such a non-specific toxic effect was unlikely and that a more subtle action involving inhibition of chondrocyte membrane transporters was probable.

Both inhibitors almost completely (>90%; Fig. 1A,B) abolished bone growth, and this emphasised their fundamental importance to bone lengthening. As both NHE1 and AE2 are involved in the control of pH_i and cell volume, it is difficult to disentangle the precise mechanism of action however by also considering the FIHC data (Fig. 5) some pertinent observations can be made. For NHE1, cell-associated labelling was initially high until approx. S5 and then fell relatively rapidly in late hypertrophy in S6 and S7 with little further change in S8. The elevated levels of NHE1 prior to, and during hypertrophy might indicate the 'housekeeping' role of this transporter to maintain optimal pH_i (Schelling & Abu Jawdeh, 2008) during cell enlargement and raised chondrocyte metabolism (Brighton *et al.,* 1973; Hunziker *et al.,* 1987). The volume of *in situ* PZ and HZ chondrocytes in EIPA-treated rudiments was significantly (approx. 3x) less (Table 2) than control cells in the same zones. However, the increase in volume of cells between these two zones was the same (approx. 2.7-fold) for both the control and EIPA-treated rudiments.

Cell volume is maintained relatively constant by the balance between membrane transporters which perform regulatory volume increase (RVI) and those that initiate regulatory volume decrease (RVD). At steady-state volume, these processes are balanced however if transporters that are involved with either of these responses are inhibited, then there is likely to be a change in cell volume (O'Neill, 1999). It is possible that this explains the reduced cell volume of chondrocytes in EIPA-treated rudiments (Table 2) as this drug will suppress NHE1 potentially leading to cell shrinkage as the RVD transporters will continue to operate. A reduction in hypertrophic chondrocyte volume is known to reduce the rate of longitudinal bone growth (Breur *et al*., 1991). It is possible therefore that the inhibitory effect of EIPA on bone growth is due to a reduction in the set-point volume of chondrocytes.

Although DIDS also inhibited the growth of bone rudiments, measurements of FIHC of AE2 along the growth plate suggested that the mechanism was different. Cell-associated levels of AE2 (Fig. 5B)

demonstrated a biphasic trend and were initially significantly lower, but then increased to section S5 corresponding to early chondrocyte enlargement. Levels then decreased following the same trend as NHE1 labelling (Fig. 5 inset). The increased levels of AE2 with hypertrophy (Fig. 5B) strongly suggested that this transporter could be involved in the transition of chondrocytes from the PZ to the HZ and it is possible therefore that DIDS inhibits rudiment growth by suppressing this phase. There was no significant difference in the volume of chondrocytes within the PZC and the HZC in rudiments cultured in DIDS (Table 2) suggesting that inhibition was not the result of preventing cell enlargement as suggested for the inhibitory effect of EIPA. It is possible that along with sustained levels of NHE1, the increased presence of AE2 during hypertrophy was required to maintain optimal pH_i in the face of the marked changes to chondrocyte metabolism occurring during hypertrophy. It was also noted that both EIPA and DIDS reduced the size of the HZ (compared to the length of the GP; Table 1). This is known to be an important determinant of bone lengthening (Wilsman *et al*., 1996b) so although the sites of actions of the drugs and their effects on cell physiology might differ, the final common inhibitory effect is the same.

The decline in levels of both transporters with late hypertrophy is notable because it has been suggested that programmed cell death ('apoptosis') in some cell types is associated with a decline in AE2 levels (Hwang *et al*., (2009), and decreased levels of NHE1 are thought to facilitate apoptotic cell death by allowing intracellular acidification which is favourable for the activity of pro-apoptotic proteins (Schelling & Abu Jawdeh, 2008). Many authors have described hypertrophic chondrocytes as dying by apoptosis (e.g. Adams & Shapiro, 2002) – the process of the programmed cell death that plays an important role in physiological cell removal, in particular during foetal development (Aigner, 2002). However, the process by which hypertrophic chondrocytes die is morphologically distinct from apoptosis e.g. cell swelling and not shrinking precedes cell death, and hence Roach *et al.,* (2004) proposed the term 'chondroptosis' to describe the non-classical apoptosis of HZ chondrocytes, which results in ultimate self-destruction of the cell without the requirement of phagocytosis.

The present results implicate a role for NHE1 and AE2 in growth plate chondrocyte hypertrophy and it is important to recognise the role of these transporters has already been established in studies on hypertrophy in other cell types. Increased protein and expression levels of NHE1 and its activation can promote hypertrophy for example in ventricular myocytes (Fliegel, 2009) and smooth muscle cells (Yu & Hales, 2011). On the other hand, pharmacological inhibition or silencing NHE1 by short interfering RNA

(siRNA) inhibited proliferation and hypertrophy (Baartscheer *et al*., 2008; Yu & Hales, 2011). There is however less information on the role of AE2 in cell proliferation and hypertrophy however the AE2(−/−) mouse suffers defective bone development/growth retardation and dies at or before weaning (Alper, 2006).

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FIGURES

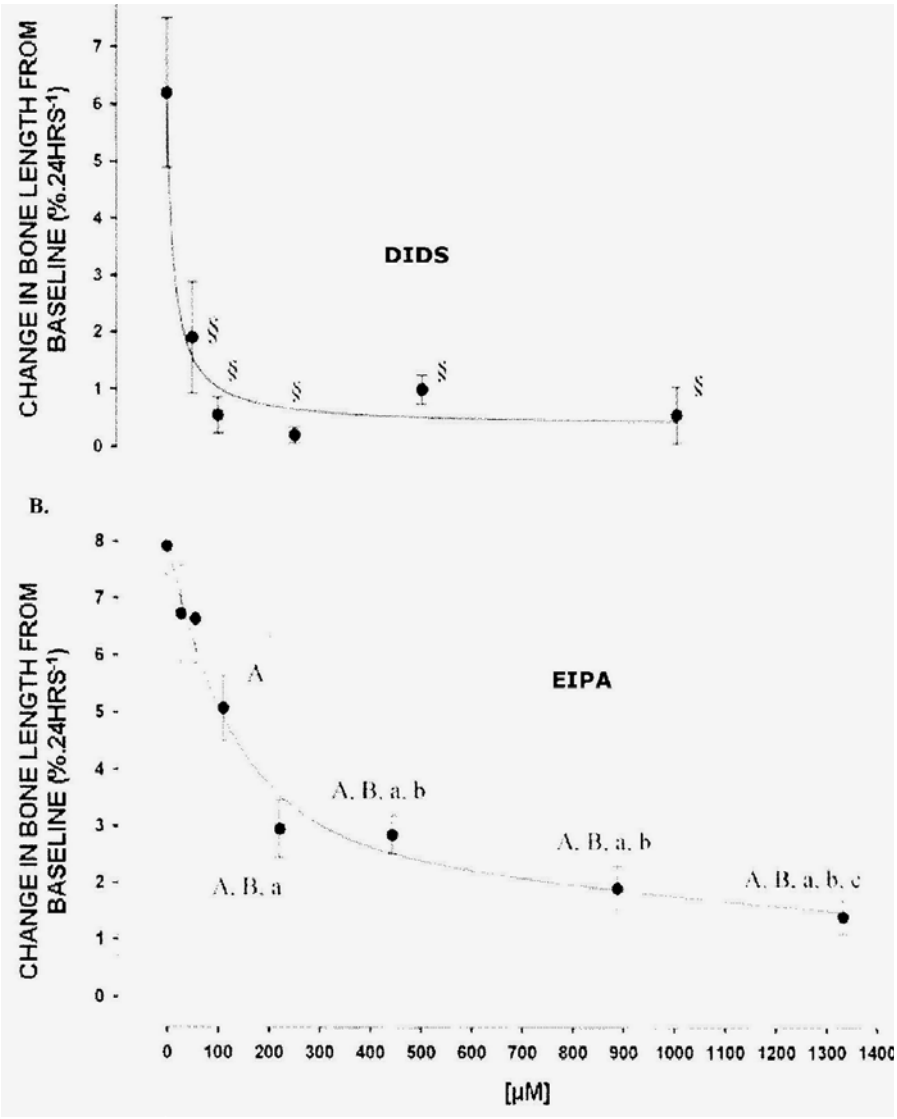


Figure 1. The inhibitory effect of (A) DIDS and (B) EIPA on rat metatarsal bone rudiment growth. DIDS or EIPA were prepared at the concentrations shown (A and B respectively), and the mean increase in bone length following culture for 24hrs determined as described (see Materials and Methods). For both drugs, there was a significant inhibition in bone growth with increasing concentration (one-way ANOVA; $P<0.001$). For all DIDS concentrations tested, there was a significant decrease from the control (no DIDS – vehicle only; §$P<0.05$; unpaired Student's t-test). For EIPA concentrations at 100µM and above, there was a significant inhibition compared to the control (unpaired Student's t-test; $P<0.05$). Data shown are means ± S.E.M. for a minimum of four metatarsals from four different animals at each drug concentration.

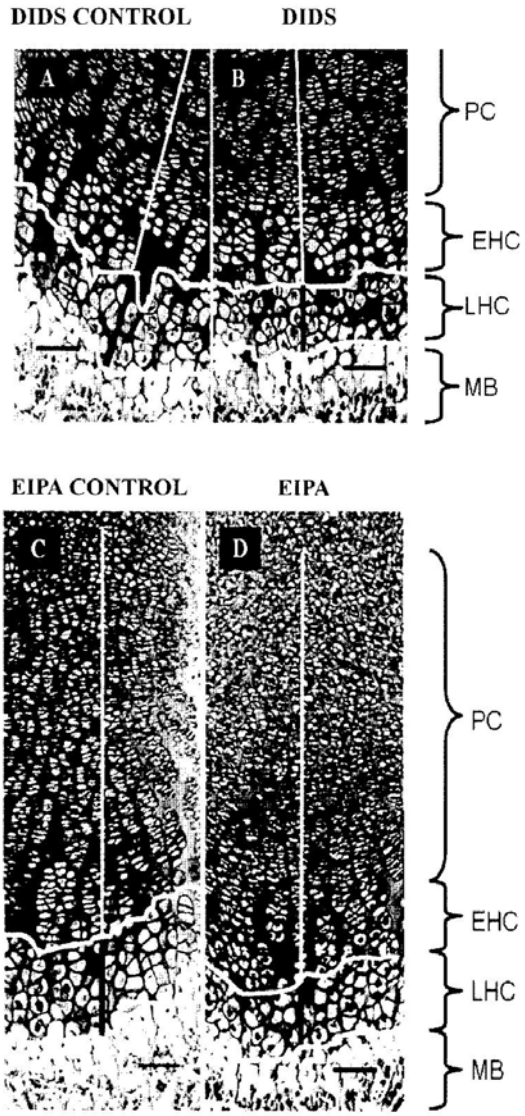
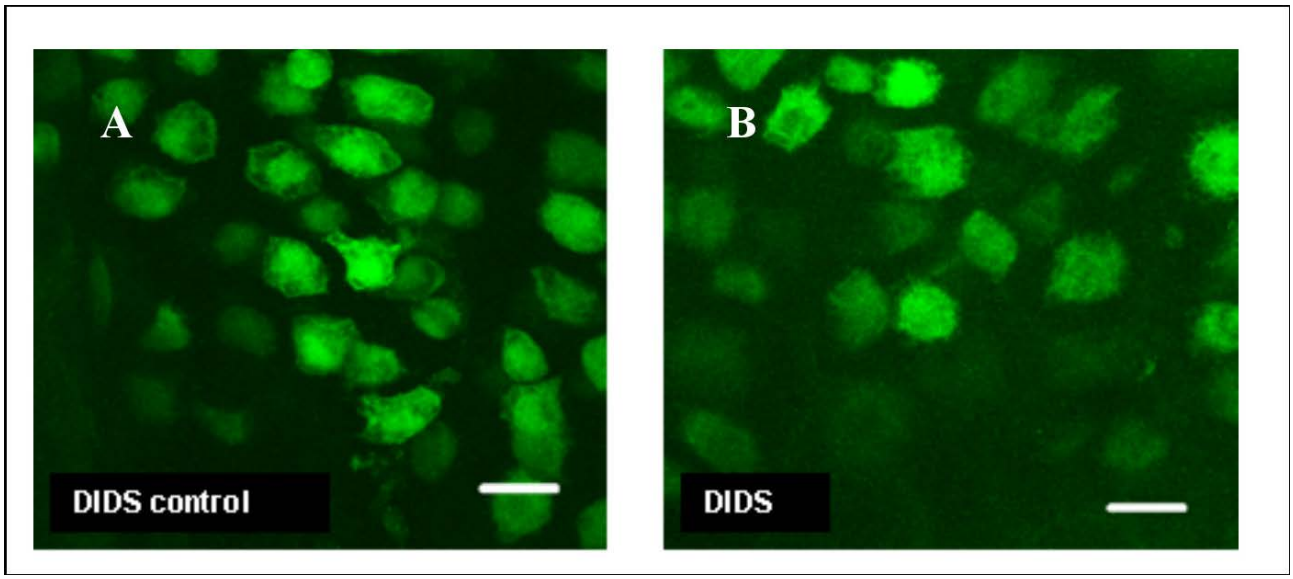


Figure 2. The height of the late hypertrophic GP zone decreased significantly in the presence of DIDS or EIPA. Following 24hrs culture with 250μM DIDS or 444μM EIPA (see Materials and Methods for details), P7 rat metatarsal bones were fixed, and sections prepared and stained with 0.1% Toluidine blue O. Using light microscopy, the proximal GP was located and the late GP hypertrophic zone cells (LHC) identified by eye in between the beginning of the late hypertrophic zone cells and the mineralized bone (MB) at the bottom (between the 2 horizontal lines). The vertical black line at the mid GP section and between the two horizontal lines indicated the average height of the late hypertrophic GP zone in DIDS and EIPA-treated bones (**B & D**), and the controls (**A & C**). The straight black and white vertical lines at the mid GP section were used to determine the total length of the GP (see Materials and Methods). PC, proliferating zone cells; EHC, early hypertrophic zone cells. Scale bar =100μm in all panels.



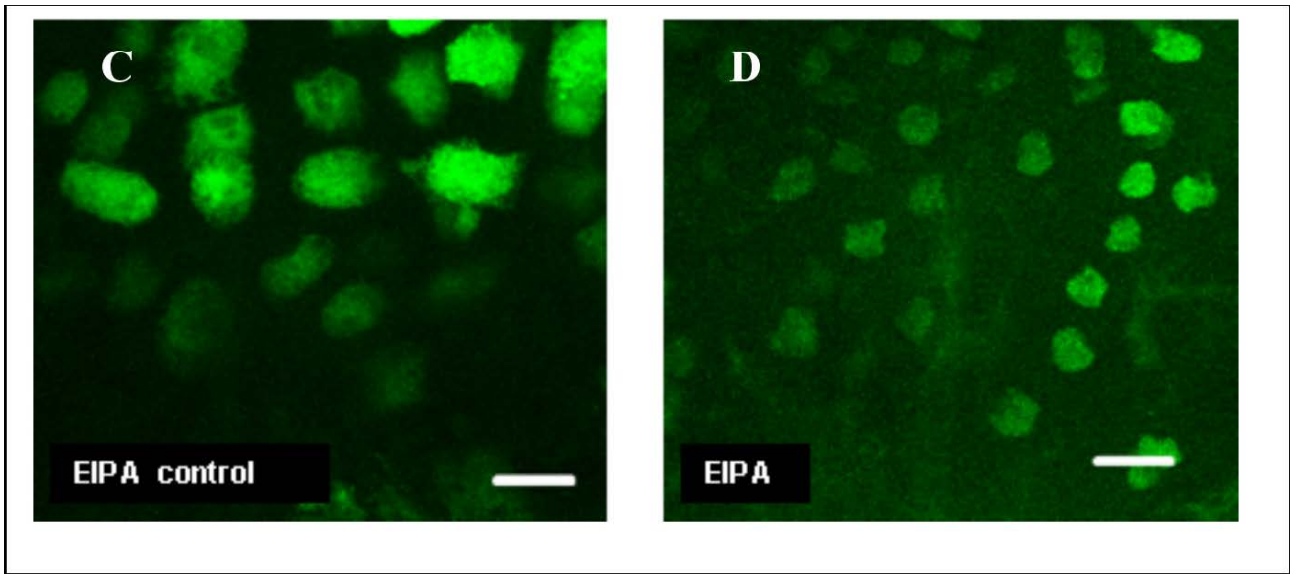


Figure 3. ***In situ*** **GPC volume after culture with DIDS (250μM) or EIPA (444μM).** Metatarsal bones were cultured in DIDS (**A control, B**) or EIPA (**C control, D)** for 24 hrs and labelled with CMFDA. Chondrocytes were then visualised by CLSM and regions of interest (ROIs) identified at the PZ and HZ, and images recorded for cell volume analysis (see Materials and Methods for details). Scale bar = 20μm in all panels.

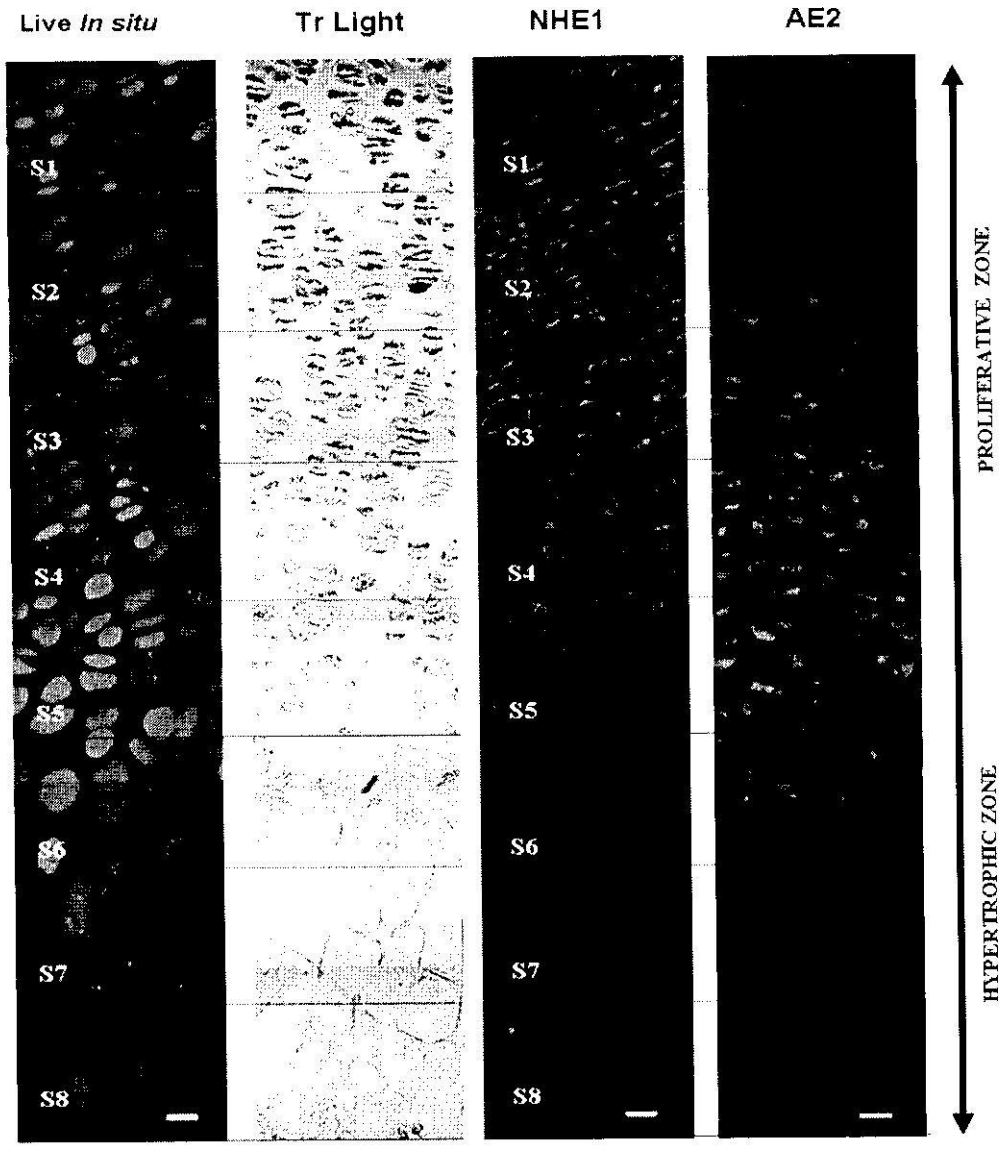


Figure 4. Positive fluorescence signal associated with NHE1 and AE2 in the GP. Paraffin-embedded sections (10μm) of proximal tibia from P7 rats showed positive fluorescence in GPC at different zone sections of proliferative zone and hypertrophic zone cells (S1-S8). Tr Light = transmitted light image corresponding to the fluorescence images. *In situ* GPC (figure far left) as viewed in unfixed live (calcein-loaded) GP tissue was shown for comparison. Images were taken using CLSM (x63 objective). Bar = 20μm.

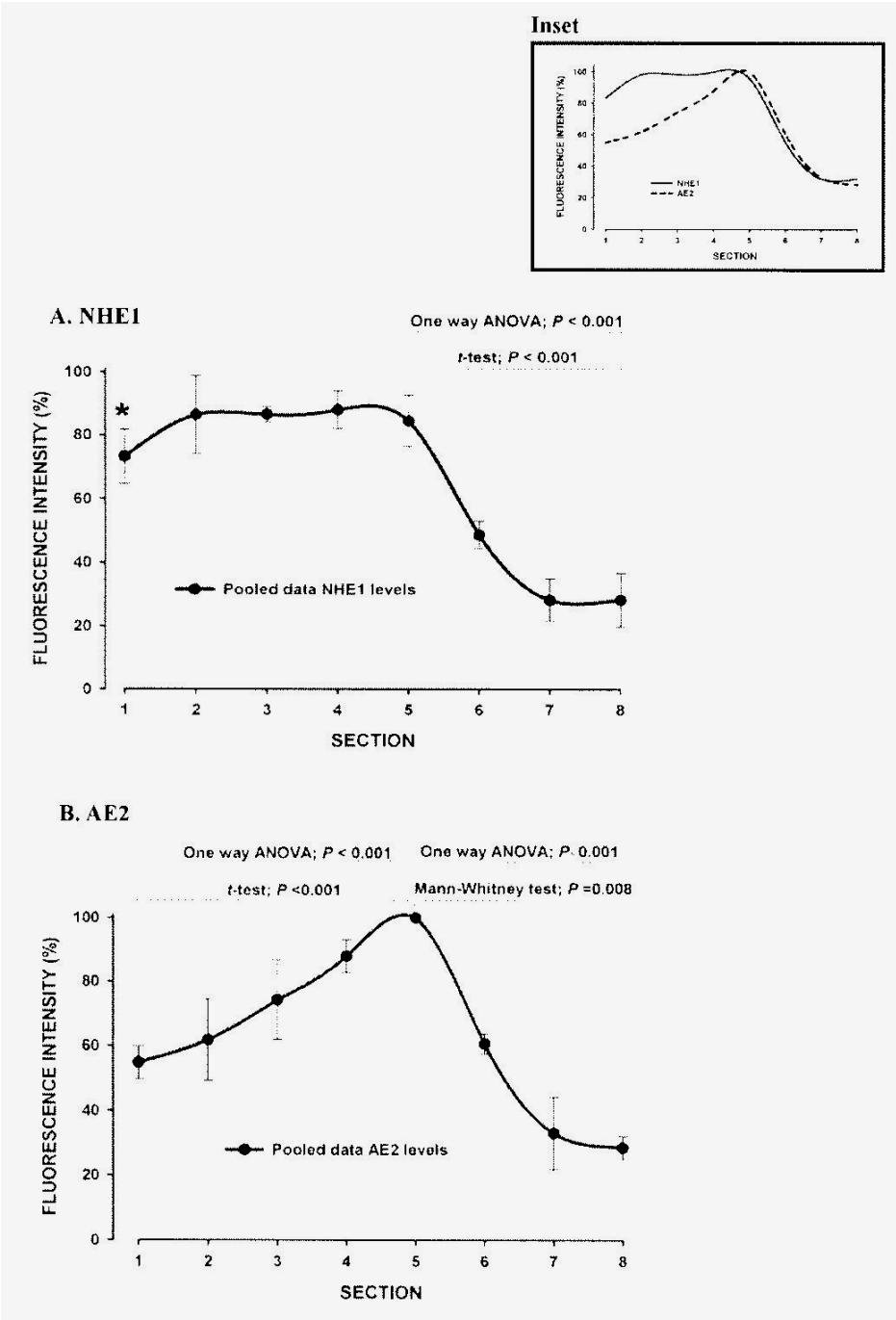


Figure 5. Localisation of NHE1 and AE2 in GPC along the GP. Chondrocyte-associated levels of (A) NHE1 and (B) AE2 fluorescence labelling are shown in the different sections along P7 rat proximal tibia GPs (see Materials and Methods for details). Data were pooled from five separate animals and at least 15 cells/section from each animal. Section 1 (S1) was nearest to the epiphyseal end and marked the early proliferative zone and section 8 (S8) was nearest to the metaphyseal end and denoted the late hypertrophic zone of the GP. The inset shows the graphs in (A) and (B) re-plotted together for comparison by normalising the data to 100% at S5. *****Significant difference compared to S1 of AE2 (Student's unpaired *t*-test; $P < 0.005$, n=5).

TABLES

Treatment (μM)	Total GP length (μm)	Late HZ length (μm)	HZ (% of total)	Late HZ cell density (cells/mm^2; n=3)
Control (n=4)	657±99	144±18	22.6±1.8	4383±25
DIDS (250μM) (n=6)	670±76	116±11	17.5±0.7*	4404±884
Control (n=5)	683±99	147±19	21.9±1.1	4007±1316
EIPA (444μM) (n=5)	668±68	117±19	17.3±1.5*	4019±1300

Table 1. The effect of DIDS and EIPA on growth plate zone height and cell density. Distal metatarsals from three to six P7 rats were cultured for 24hrs in the absence/presence of DIDS or EIPA at the concentrations indicated. Bones were fixed, prepared, and visualized as described in Materials and Methods. There was significant reduction in height (%) of hypertrophic zone (HZ) from the total height of the growth plate in DIDS or EIPA-treated bones. However the average height of the growth plates and hypertrophic zone between control and treated bones were not significantly different. Hypertrophic zone cell density of treated bones also showed no significant difference from the control. *Significantly different from the corresponding control (P<0.05; unpaired Student's t-test). (Data are means ± S.E.M. for n experiments on separate animals).

Treatment (µM)	GPC volume (µm^3 from (n[N])	
	PZC (S1 – S2)	**HZC (S7 – S8)**
Control	609±46 (6[29])	1880±230 (6[44])
DIDS (250)	572±71 (3[25])	2660±419 (3[27])
Control	761±50 (6[33])	2044±219 (6[38])
EIPA (444)	211±26* (3[15])	586±54* (3[19])

Table 2. The effect of EIPA and DIDS on *in situ* growth plate chondrocyte volume. Distal metatarsals from three to six P7 rats were cultured for 24 hrs in the absence/presence of DIDS or EIPA at the concentrations indicated. Bones were bisected, loaded with fluorescent dye (CMFDA), fixed and visualized using the CLSM technique as described in Materials and Methods. Data are means ± S.E.M. *Significant difference from the control (P<0.01; unpaired Student's t-test).